PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Synopses from the Poster Exhibition Organized by R. H. Pain. 1. New Very Efficient Antibiotics in the Field of Cephalosporin Derivatives

R. Bucourt, R. Heymes, A. Lutz, L. Penasse and J. Perronnet

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1. New very efficient antibiotics in the field of cephalosporin derivatives

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The semi-synthetic cephalosporin derivatives containing a 2-(2-amino-4-thiazolyl)-2-*syn*-oxyiminoacetyl side chain are obtained according to the general reaction sequence outlined in figure 1. They are characterized by a very high level of antimicrobial activity, especially against Gram-negative species. This unexpected result is due to the conjunction of two structural features: the aminothiazolyl moiety and the *syn*-oxyimino substituent.

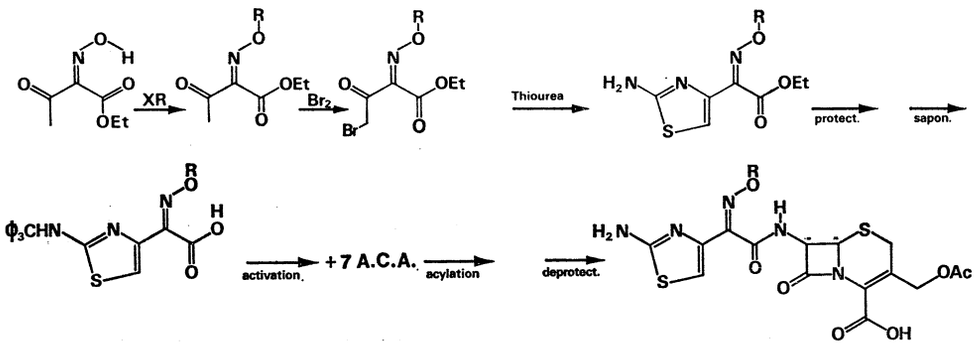
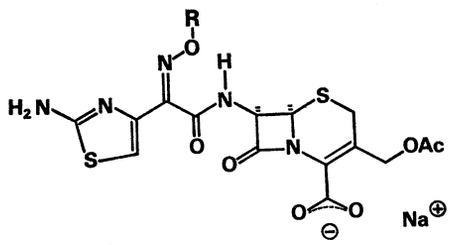


Figure 1



R = Me = Cefotaxime = HR 756
Et
iPr
H = HR 111

Compounds **1**. Alkoxyimino derivatives.

When compared among themselves (table 1) the compounds **1** seem about equivalent with regard to the Gram-positive bacteria. Against Gram-negative species their level of antimicrobial activity significantly decreases as the size of the alkoxy group increases. This suggests that a greater lipophility is unfavourable. Therefore, the *syn*-methoxyimino derivative, Cefotaxime (= HR 756), was selected for development.

However, the hydroxyimino derivative, HR 111, exhibits some lower minimum inhibitory concentration (m.i.c.) values than Cefotaxime in Gram-positive species, whereas it possesses the same very good activity against Gram-negative organisms (except *P. vulgaris*). These

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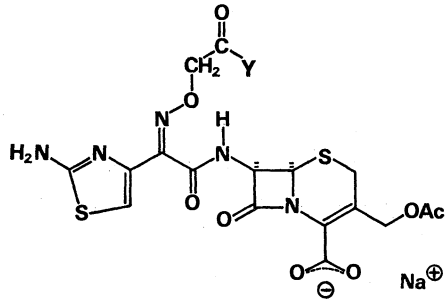
TABLE 1. ANTIMICROBIAL ACTIVITY OF CEPHALOSPORIN DERIVATIVES

		S. aureus, ATCC 6538	*S. aureus*, Institut Pasteur 54146	*S. aureus*, penicillin resist.+	*S. faecalis*, Institut Pasteur 5432	*B. subtilis* ATCC 6633	*E. coli* ATCC 11303	*E. coli* TO 26 B 6	*E. coli*, ATCC 9637	*K. pneumoniae*, NCTC 5055	*K. pneumoniae*, R 2536	*S. typhimurium*	*P. mirabilis*, Institut Pasteur A 235	*P. vulgaris*, Institut Pasteur A 232	*Providencia* sp.	*S. marcescens*
	cefazolin	0.2	0.6	1	20	0.1	2	2	2	2	30	2	5	> 50	> 50	> 50
	cefamandole	0.4	0.4	0.6	40	0.05	0.4	1	1	0.1	50	1	2	> 50	> 50	> 50
compounds **1**																
R = Me	cefotaxime	1	2	2	2	0.5	0.02	0.1	0.2	0.05	0.5	0.1	0.02	10	5	0.5
R = Et	—	2	2	2	2	0.4	0.2	0.4	1	0.05	2	1	0.05	1	5	2
R = iPr	—	2	2	3	5	0.5	0.5	1	2	0.2	10	0.5	0.5	0.5	5	2
R = H	HR 111	0.5	0.5	0.5	3	0.2	0.02	0.1	0.05	0.02	0.1	0.05	0.05	40	3	0.4
compounds **2**																
Y = OEt	—	3	3	3	5	0.5	0.5	1	2	0.1	2	1	0.2	1	5	5
Y = NH_2	—	2	3	5	5	0.5	0.05	0.1	0.2	0.05	0.5	0.2	0.1	5	5	2
Y = ONa	HR 097	10	20	20	20		0.05	0.1	0.2	0.1	0.1	0.3	0.02	0.1	1	1

Results obtained with serial dilution test in Muller–Hinton broth inoculated with approximately 10^5–10^6 colony-forming units/ml.

results, supported by cumulative m.i.c. distributions, prompted us to investigate rather polar substituents for the oxyimino group.



Y = OEt (low activity)
NH_2
O^-Na^+ = HR 097

COMPOUNDS **2**. (2-Oxoethoxy)imino derivatives.

Among the compounds **2** the carbethoxy derivative is the least active against Gram-negative species (table 1), whereas the amidic and the carboxylic (HR 097) derivatives display an efficient activity in this field. It should be noted that the shape of the antimicrobial spectrum is quite different from that of HR 111. HR 097 is specifically active against Gram-negative organisms and sometimes (*P. vulgaris*) more potent than Cefotaxime.

Thus substitutions on the oxyimino group introduce various spectrum alterations: (i) a decrease in Gram-negative antibacterial activity is observed when the carbon atom number increases; (ii) an increase in some Gram-positive antibacterial activity is obtained with the hydroxyimino compound HR 111; (iii) an increase in some Gram-negative antibacterial activity with simultaneous decrease in activity against Gram-positive bacteria is noticed with the carboxylic derivative HR 097.

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2. Biosynthesis of penicillins and cephalosporins in cell-free systems

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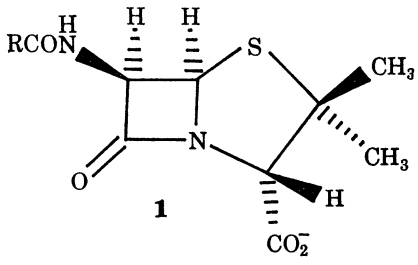
Penicillium chrysogenum produces penicillins with non-polar side chains, such as benzylpenicillin (**1*a***, figure 1), and also small amounts of isopenicillin N (**1*b***) which has a δ-linked L-α-aminoadipyl side chain. *Cephalosporium acremonium* and certain *Streptomyces* species excrete only one penicillin (penicillin N, **1*c***) and various cephalosporins (**2*a*** to **2*f***, figure 1), all of which have a D-α-aminoadipyl side chain. Several cephalosporins produced by *Streptomyces* species contain a 7α-methoxy group.

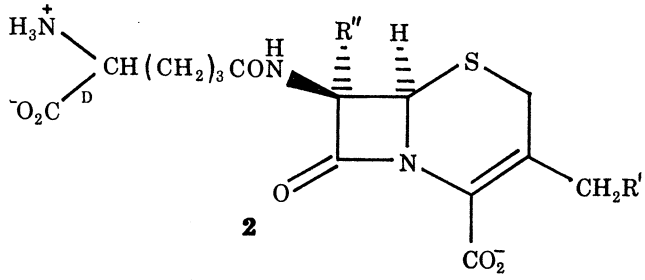
The amino acid precursors of these substances were readily identified by feeding isotopically labelled amino acids to the intact organisms, but potential intermediates could not be so tested because they were not transported into the antibiotic-producing cells. The first cell-free system in which synthesis of the penicillin ring system could be demonstrated was prepared by Fawcett *et al.* (1973) by lysis of protoplasts of *C. acremonium*. In this system, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (but not its LLL or DLD isomer) was a precursor of a penicillin with an α-aminoadipyl side chain (Fawcett *et al.* 1976).

The LLD tripeptide has now been synthesized with ^3H in both its α-aminoadipyl and valine residues and has been shown to be incorporated with its carbon skeleton intact into the penicillin formed in the cell-free system. This penicillin is mainly isopenicillin N, because about 85 % of the α-aminoadipic acid obtained from it by hydrolysis behaves as the L-isomer to L-amino acid oxidase (O'Sullivan *et al.* 1979*b*). It is thus likely that isopenicillin N is the immediate precursor of the penicillin N excreted by *C. acremonium*. Penicillin N is believed to be a precursor of deacetoxycephalosporin C (**2*a*** in figure 1; Yoshida *et al.* 1978). Moreover, isopenicillin N is converted to benzylpenicillin by an acyl transferase present in an extract of *P. chrysogenum* (A. J. Fisher & E. P. Abraham, unpublished). Hence, isopenicillin N appears to occupy a central position in penicillin and cephalosporin biosynthesis.



1

penicillins	R
(*a*) benzylpenicillin	$C_6H_5CH_2$
(*b*) isopenicillin N	$^-O_2C\cdot CH(\overset{+}{N}H_3)\cdot CH_2\cdot CH_2\cdot CH_2$ (L)
(*c*) penicillin N	$^-O_2C\cdot CH(\overset{+}{N}H_3)\cdot CH_2\cdot CH_2\cdot CH_2$ (D)



2

cephalosporins	R′	R″
(*a*) deacetoxycephalosporin C	H	H
(*b*) deacetylcephalosporin C	OH	H
(*c*) cephalosporin C	$OCOCH_3$	H
(*d*) 7α-methoxycephalosporin C	$OCOCH_3$	OCH_3
(*e*) *O*-carbamoyl deacetylcephalosporin C	$OCONH_2$	H
(*f*) cephamycin C	$OCONH_2$	OCH_3

FIGURE 1. Structures of biosynthetic penicillins and cephalosporins.

Mass spectrometric analysis of a 7-α-methoxycephalosporin (cephamycin C, **2*f***) produced by *Streptomyces clavuligerus* in the presence of $^{18}O_2$ has demonstrated that the oxygen of the methoxy group is derived from molecular oxygen (O'Sullivan *et al.* 1979*a*). We have now shown that the 7-α-methoxylation of cephalosporin C (**2*c***) and its carbamoyl analogue (**2*e***) can be accomplished in a cell-free system prepared by brief sonication of *S. clavuligerus*, but that the

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methoxylation of deacetoxy- and deacetylcephalosporin C does not occur to a detectable extent under these conditions. The reaction has been followed by use of *S*-adenosyl-[methyl-^{3}H]-methionine as a methyl donor and by measurement of the ^{3}H in cephamycin C after isolation of the latter by electrophoresis on paper. Since the reaction is dependent on the presence of Fe^{2+}, 2-oxoglutarate and a reducing agent such as ascorbate or dithiothreitol, the oxidative step is catalysed by an enzyme that has properties of a dioxygenase. The results indicate that the methoxy group of these 7-α-methoxycephalosporins is introduced after the formation of the cephalosporin ring system and after the CH_3 at C-3 of deacetoxycephalosporin C has been converted to CH_2OCOR, where R is CH_3 or NH_2.

Stages in the biosynthesis of penicillins and cephalosporins that are consistent with the findings reported here are shown in figure 2.

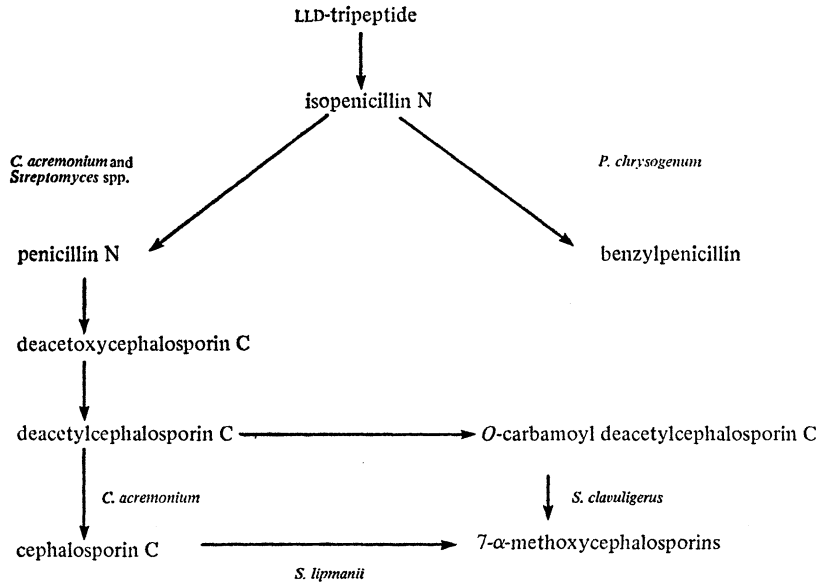

FIGURE 2. Stages in the biosynthesis of penicillins and cephalosporins.

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3. Cooperation of benzylpenicillin and cefoxitin in bacterial growth inhibition

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The Gram-negative bacterium *Proteus mirabilis* is distinguished by a partial 'intrinsic' resistance to β-lactam antibiotics. This type of resistance has been observed in different bacteria. Its mechanism is still unknown. It is presumed to be due to an inherently low sensitivity of a vital

bacterial target to β-lactam action (Sabath, this symposium). Thus it is different from two other types of resistance, inactivation of β-lactams by β-lactamases, and penetration resistance due to the shielding of targets from β-lactams by a penetration barrier on the cell surface. Cells of *P. mirabilis* are converted to spheroplasts when grown in the presence of various β-lactam antibiotics added individually to the growth medium at high concentration. Cell walls of the spheroplasts are deformed and fragile, an obvious consequence of functional damage in the cell wall peptidoglycan. However, this event is not lethal. With 200 units/ml (120 μg/ml) benzylpenicillin, for instance, the organism grows vigorously in the so-called spheroplast type L-form (figure 1), and peptidoglycan continues to be synthesized in the spheroplast walls. In the chemical

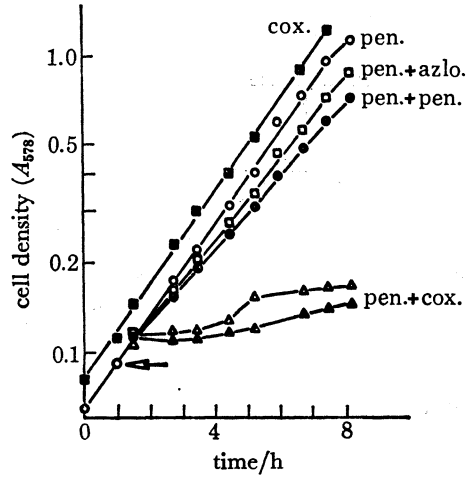

FIGURE 1. Growth curves of spheroplast type L-form of *P. mirabilis* in shake culture with benzylpenicillin (pen., 120 μg/ml) or cefoxitin (cox., 150 μg/ml). Different cultures started with pen. received an additional β-lactam, azlocillin (azlo., 100 μg/ml), pen. (100 μg/ml) or cox. (upper curve, 10 μg/ml; lower curve, 100 μg/ml) at the time indicated by arrow.

composition of this peptidoglycan one cannot detect the change in peptide cross-linkage and D-alanine content (Martin & Gmeiner 1979) that one would expect as consequences of the known inhibitory action of β-lactams on specific target enzymes of peptidoglycan synthesis (Blumberg & Strominger 1974), namely peptidoglycan transpeptidase (TPase) and DD-carboxypeptidase (CPase). Therefore, the closely related functions of TPase and CPase, which may be alternative reactions of the same enzyme (Ghuysen 1976), must remain operative, at least in part, during the partially β-lactam-resistant state of *P. mirabilis*. Studies on the interaction of isolated target enzymes from *P. mirabilis* with β-lactam antibiotics provide the key for understanding some aspects of intrinsic β-lactam resistance and suggest how it may be overcome.

Two different, membrane-bound DD-carboxypeptidases named L and H (comparable with CPase 1A and 1B from *Escherichia coli*; Tamura *et al.* 1976) were isolated from *P. mirabilis* and purified (Martin *et al.* 1976; Schilf 1978). In the highly purified state, DD-carboxypeptidase H and DD-carboxypeptidase L functioned as transpeptidases *in vitro* with the pentapeptide of the natural peptidoglycan precursor as substrate. Enzymes L and H were also characterized as penicillin-binding proteins (PBPs; Spratt 1978; Ohya *et al.* 1978) number 5 and 4, respectively, among seven PBPs present. With benzylpenicillin only CPase–TPase H was permanently inhibited with a half-life $T_{\frac{1}{2}} = 300$ min for the enzyme–inhibitor complex EI* (Schilf 1978). In contrast, EI* formed from benzylpenicillin and CPase–TPase L decayed rapidly ($T_{\frac{1}{2}} = 3.5$ min)

with liberation of reactivated enzyme and degradation of the antibiotic to benzylpenicilloic acid (Martin *et al.* 1976; Schilf *et al.* 1978). Thus it can be deduced that CPase–TPase activity found in L-form spheroplasts grown with benzylpenicillin was due to the presence of CPase–TPase L only in enzymically active form. Also, in such spheroplasts PBP 5 remained as the only prominent penicillin binding protein (in addition to PBPs 2 and 6 of unknown enzymic function).

A search was carried out among commercially available β-lactam antibiotics to detect a compound capable of inhibiting L-form growth when added to cultures growing with benzylpenicillin (figure 1). It was assumed that such a β-lactam would permanently inactivate those vital targets that benzylpenicillin could inhibit only transiently. The great majority of β-lactams tested (e.g. azlocillin) were not effective. Cefoxitin, however, was able specifically to inhibit L-form growth in combination with benzylpenicillin. Cefoxitin was also specific in causing permanent inactivation ($T_{\frac{1}{2}} = 900$ min) of the functional CPase–TPase L in the spheroplasts grown with benzylpenicillin. Finally, the combined action of benzylpenicillin and cefoxitin resulted in a 50% decrease of peptide cross-linkage in the peptidoglycan. Good L-form growth occurred with cefoxitin alone. Spheroplasts grown with cefoxitin contained normally cross-linked peptidoglycan. Functional CPase–TPase in such spheroplasts was enzyme H only, which formed a labile complex with cefoxitin ($T_{\frac{1}{2}} = 14$ min). As expected, CPase–TPase H was also revealed as PBP 4 among the residual penicillin binding proteins in spheroplasts grown with cefoxitin, together with vastly reduced quantities of PBPs 5 and 6 and some higher molecular mass PBPs of unknown enzymic function.

Conclusion

In *Proteus mirabilis* no single β-lactam antibiotic of the presently available commercial compounds can carry out *permanent* inactivation of the multiple β-lactam targets which are individually competent to ensure viability and synthesis of peptide cross-linked peptidoglycan. Partial intrinsic resistance to a single β-lactam results from the continued function of one of the target enzymes, which the antibiotic in question can inhibit only transiently. This resistance can be overcome by simultaneous application of a *suitable* pair of β-lactams which cooperate in the permanent inactivation of all essential targets.

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4. The action of some β-lactam antibiotics on the penicillin-binding proteins of Gram-negative bacteria

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The antibacterial activity of β-lactam antibiotics against Gram-negative bacteria depends on three interacting parameters. First, the antibiotics must be able to bind to and inhibit 'essential' penicillin-binding proteins located in the cytoplasmic membrane; secondly, the compounds must be able to penetrate the permeability barrier of the outer membrane of the bacterial envelope; thirdly, the antibiotics must be resistant to any periplasmically located β-lactamases. Strains of Gram-negative bacteria not elaborating either an inducible or an R-factor-mediated β-lactamase owe their resistance to β-lactam antibiotics primarily to the first two considerations.

Recent work from a number of laboratories has shown that at least seven distinct penicillin-binding proteins (PBPs) can be consistently detected in membranes of *E. coli* K12. These proteins (numbered 1a, 1b, 2, 3, 4 and 5/6 in order of decreasing molecular mass) covalently bind β-lactam antibiotics with varying affinities depending on the PBP and antibiotic concerned.

Some of these PBPs have been shown to perform 'essential' functions in bacterial elongation (PBPs 1a/1b), shape determination (PBP 2) and division (PBP 3) whereas others (PBPs 4 and 5/6) appear to be 'dispensible'. β-Lactam antibiotics exert their lethal effects upon *E. coli* by binding to one or more 'essential' PBPs, thereby inhibiting their functions in morphogenesis.

The isolation and analysis of temperature-sensitive mutants with lesions in the structural genes for the various 'essential' PBPs, has demonstrated that PBPs 1a/1b are involved in the biosynthesis of peptidoglycan associated with bacterial elongation such that their joint inhibition results in spheroplast formation and rapid lysis. PBPs 2 and 3 have likewise been shown to perform 'essential' functions in determining the rod morphology and in the cell division process of *E. coli* K12 respectively. Consequently, inhibition of PBP 2 causes *E. coli* to grow as osmotically stable round-forms and blocking of PBP 3 results in filamentation. As with inhibition of PBPs 1a/1b, inhibition of either PBP 2 or PBP 3 is lethal to the bacteria although the 'physiological mechanism' through which the bacteria die may be different and perhaps somewhat less rapid. The remaining PBPs, 4 and 5/6, are believed to be 'dispensible' and to play little part in the antibacterial action of β-lactam antibiotics since mutants with defects in these proteins are perfectly viable.

It has been realized for some time that different β-lactam antibiotics, or even different concentrations of any one antibiotic, can cause different but distinct morphological effects in *E. coli* K12. These morphological effects can be specifically correlated with the differential inhibition of discrete 'essential' PBPs, in a manner that directly parallels the behaviour of the appropriate temperature-sensitive mutants at their non-permissive temperatures.

The competition of a wide range of both commercial and novel β-lactam antibiotics for the PBPs of *E. coli* DC0 has been studied and the results compared with the effects of the compounds upon morphology and with their antibacterial activity against both *E. coli* DC0 and its isogenic 'permeability' mutant, *E. coli* DC2. The affinities of the compounds for the PBPs of *E. coli* DC0 have been determined by competition with [^{14}C]benzylpenicillin by the procedure of Spratt.

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Forty different β-lactam compounds have been examined and it has been confirmed that different antibiotics show different affinities for the PBPs of *E. coli* DC0 and that close correlation can be seen between the affinities of the compounds for their primary 'essential' PBP target and the morphological effects that they induce. For example, cephaloridine and cefsulodin show highest affinity for PBPs 1a/1b and cause spheroplasting and lysis, whereas cefuroxime, cefotaxime, cefamandole and mezlocillin have highest affinity for PBP 3 and cause filamentation.

Since the permeability barrier present in intact growing bacteria is not operative in the PBP assay, a comparison of the affinities of the various antibiotics for their primary 'essential' PBP targets with their antibacterial activity against growing cultures of *E. coli* DC0 is an indication of the ability of the antibiotics to penetrate the outer membrane permeability barrier.

On this basis, we have observed that different β-lactam antibiotics are excluded to a greater or lesser degree. For example, cephaloridine inhibits PBPs 1a/1b (its primary target) at about the same concentration as its minimum inhibitory concentration (m.i.c.) suggesting that this compound is not hindered by the outer membrane. On the other hand, cefuroxime, cefotaxime, cefamandole and mezlocillin inhibit PBP 3 (their primary target) at concentrations significantly lower than their m.i.cs against *E. coli* DC0, implying that these compounds are retarded by the outer membrane in this organism. In support of this we have found that the above compounds, and indeed all the compounds tested, have m.i.cs against the 'permeability' mutant *E. coli* DC2 that are in close agreement with the affinities of the antibiotics for their primary 'essential' PBP targets, irrespective of whether that target is PBPs 1a/1b, PBP 2 or PBP 3.

Examples of this phenomenon that highlight the usefulness of the PBP assay as a 'cell-free' system for evaluating β-lactam antibiotics are provided by methicillin and cloxacillin. Both of these antibiotics have m.i.cs against *E. coli* DC0 in excess of 250 μg/ml but nevertheless inhibit PBP 3 (their primary target) at about 6 and 3 μg/ml respectively. This implies that if these compounds could overcome the permeability barrier of *E. coli* DC0 they would have useful antibacterial activity. The fact that both compounds have m.i.cs against *E. coli* DC2 of about 4 μg/ml substantiates this hypothesis. Furthermore, both compounds cause filamentation in *E. coli* DC2, as expected from their PBP affinities.

A number of penicillin and cephalosporins are known that have higher or exclusive affinities for different 'essential' PBPs in *E. coli* K12 and consequently induce distinct morphological effects. Such compounds can thus be used as morphogenic probes of PBP function in other Gram-negative bacteria where they also induce the same morphological effects. We have studied the competition of four such probes (cephaloridine, mecillinam, cefuroxime and cefoxitin) for the PBPs of *E. coli*, *E. cloacae*, *K. aerogenes*, *Pr. rettgeri* and *Ps. aeruginosa*. All of the strains used are mutants that expressed their respective chromosomal β-lactamases at no more than 0.1 units/mg dry mass of bacteria and that are also non-inducible. At this level of expression, the β-lactamases play little part in the resistance of the bacteria to penicillins and cephalosporins.

The different Gram-negative bacteria show similar, but not identical, PBP patterns, with species-specific variations in both PBP molecular mass and in the relative proportion of the different PBP bands.

Comparison of the affinities of the four morphogenic probes for the PBPs of the different strains with their morphological effects indicates that the PBPs of the different organisms perform the same functions in morphogenesis as they do in *E. coli* K12. In all cases, cephaloridine has highest affinity for PBPs 1a/1b and causes spheroplasting, mecillinam binds exclusively to

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PBP 2 and causes round-form production, and cefuroxime has highest affinity for PBP 3 and causes filamentation. Cefoxitin shows high affinity for the 'non-essential' PBPs 4 and 5/6 in all of the Gram-negative strains studied, and little affinity for PBP 2.

By using the PBP affinity data to gauge the penetration characteristics of the β-lactam antibiotics in the different Gram-negative bacteria, as described earlier for *E. coli* DC0, we have found little difference between *E. coli*, *E. cloacae*, *K. aerogenes* and *Pr. rettgeri*, at least for the four probe compounds tested.

However, a different situation exists in *Ps. aeruginosa*. With this organism, all four compounds have m.i.cs significantly in excess of their 'primary' binding concentrations. For example, cephaloridine inhibits PBPs 1a/1b of *Ps. aeruginosa* at about 4 μg/ml compared with an m.i.c. of 125 μg/ml, whereas in *E. coli* this antibiotic inhibits PBPs 1a/1b at around 2 μg/ml, the same concentration as its m.i.c. The data indicate that the more impermeant nature of the envelope of *Ps. aeruginosa* is a major factor in the resistance of this organism to β-lactam antibiotics.

In the search for a new generation of β-lactam antibiotics with improved therapeutic efficacy, a much more detailed understanding of the various interacting parameters of 'target-activity', permeability and β-lactamase stability which together determine the resistance 'phenotype' of Gram-negative bacteria, is necessary. The usefulness of the penicillin-binding protein assay and 'permeability' mutants in this context is apparent and could perhaps provide a means of elucidating the structure–activity relations necessary for the rational design of β-lactam antibiotics, which combine the optimal characteristics for chemotherapeutic efficiency.

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5. Active site of staphylococcal β-lactamase

By S. J. Cartwright and A. F. W. Coulson
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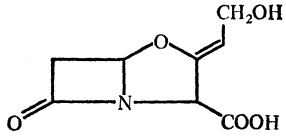
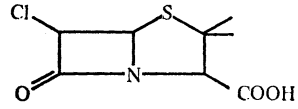
We describe here the design and synthesis of an active-site-directed inhibitor of the penicillinase from *Staphylococcus aureus*, and its use to identify active site residues in this enzyme.

Our starting point was a study of the interaction of the purified exocellular enzyme with clavulanic acid, **1**. Clavulanic acid is a fermentation product, and our sample was a generous gift from Beecham Pharmaceuticals.



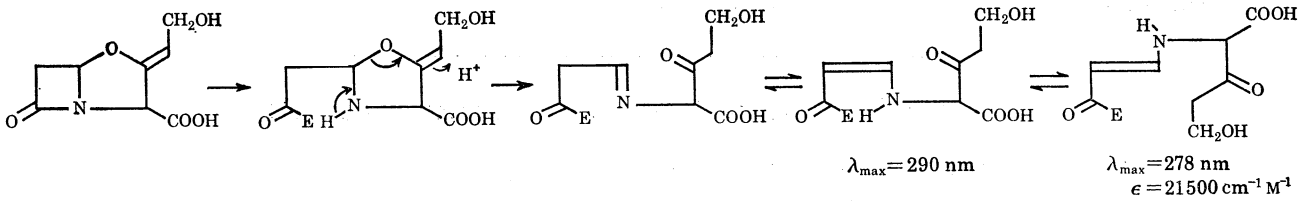
1



2

Clavulanate inactivates the staphylococcal enzyme by one-to-one covalent reaction with the active site. The apparent K_m is 12 μM, and the enzyme can be protected by substrates and competitive inhibitors. The reaction product was characterized by observation of its UV spectrum, and principally on this basis (together with experiments with model compounds), scheme 1 was proposed as the mechanism of the interaction.

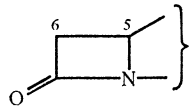
The essential part of this suggestion is that an acyl enzyme on the normal hydrolytic reaction pathway rearranges to give finally a 2-amino-1,2-unsaturated acyl-enzyme which, unlike the saturated acyl-enzymes derived from normal substrates, is only slowly hydrolysed. Other routes to the same product, and further decomposition (for example decarboxylation) of the attached residue, are compatible with the present evidence.



SCHEME 1

It is postulated that the acid-catalysed reactivation of the enzyme occurs by hydrolysis of the Schiff base form of the inhibitor fragment. This process is too fast even in neutral solution to make isolation of labelled peptides easy, and we therefore sought an inactivator with a similar mechanism but giving a more stable product (Cartwright & Coulson 1979).

According to scheme 1, the critical difference between inactivators and substrates is the possibility of elimination across the C-5–C-6 bond. This requires a good leaving group at C-5 and a sufficiently acidic proton at C-6.



Accordingly, we synthesized chloropenicillanic acid sulphone (**2**) in two steps from 6-aminopenicillanic acid. This compound proved to be a substrate and inactivator of the enzyme. The inactivated form of the protein is similar to that derived from clavulanate, but the label is much more firmly attached, and isolation of peptides is straightforward provided the pH is kept above 8.0.

Inactivated protein was digested with chymotrypsin, and a single labelled peptide (identified by the characteristic u.v. spectrum of the attached inhibitor fragment) isolated by gel filtration and DEAE chromatography. The peptide (residues 69–80) was redigested with trypsin, and a single labelled pentapeptide isolated and sequenced. The sequence is Ala-Ser-Thr-Ser-Lys (residues 69–73). Redigestion with pronase and isolation of fragments gave a labelled Ser-Thr dipeptide.

Ser 70 is homologous with that labelled by β-bromopenicillanate in the *Bacillus cereus* enzyme (Knott-Hunziker *et al.* 1979), and the mechanism proposed for this inactivation is analogous to that in scheme 1. Professor Knowles (this symposium) describes the isolation of a homologous peptide from the R_{TEM} enzyme treated with a penicillin sulphone, and gives evidence that inhibition by these compounds and by penicillanic acid sulphone (English *et al.* 1978) proceeds by a similar mechanism.

The work described here has identified part of the active site of the penicillinase from *S. aureus*. It has also provided a specific suggestion of part at least of the mechanism of inactivation by clavulanate. It has demonstrated the feasibility of a rational approach to the design of inactivators for this group of enzymes. Finally, it gives support to the possibility that the catalytic

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activity of the enzyme has a mechanism analogous to that of the serine proteases, involving the obligatory intermediary of an acyl enzyme.

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6. Reversible deactivation of β-lactamase by penicillin analogues

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Certain semi-synthetic penicillins are poor substrates for β-lactamases and may be divided into two classes, one being simply poor substrates, the other actually bringing about a deactivation of the enzyme to a low activity state. This latter phenomenon of substrate-induced deactivation has been shown to be fully reversible (Virden *et al.* 1978) and is quite different from the irreversible, chemical inactivation in the R_{TEM}–clavulanate system (Fisher *et al.* 1978). The reversible deactivation is characterized by a biphasic time course of activity, involving a transition from a form with high activity against benzylpenicillin to one showing lower activity against the same substrate. The transition is first-order with a characteristic half-time $t_{\frac{1}{2}}$ = 10–30 s. These kinetics have been modelled satisfactorily, assuming a simple, slow interconversion between two forms of the enzyme or enzyme substrate complex:

$$\mathrm{E}+\mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E}+\mathrm{P},$$
$$\mathrm{ES} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E^*S}$$

where k_{-3} is appreciably slower than k_2.

The question arises as to the nature of E*S (or E*). Early work suggested a changed conformation in the enzyme, on the basis of changed sensitivity to iodine and to urea. Further evidence for conformational change was provided by Citri *et al.* (1976), who showed that certain antisera directed against the enzyme can lock it into a high-activity form, thus relieving the biphasic nature of the kinetics. Sulphate ions, which stabilize the native state of the enzyme, will also 'lock-in' the enzyme to its higher-activity state (Mitchinson & Pain 1980).

The conformational basis of the response of the β-lactamase from *S. aureus* has now been fully established by immunological criteria. Quinacillin, when incubated with the enzyme as described above, induces a low-activity state in the enzyme within two minutes. When an aliquot is diluted into benzylpenicillin solution, the enzyme hydrolyses this substrate at a low initial rate but, when all of the quinacillin has been hydrolysed, the enzyme returns to its full benzylpenicillinase activity. A precipitating antiserum was chosen which only reduces the activity of the β-lactamase to 60 % of its native activity. Addition of this antiserum to the enzyme preincubated in quinacillin, and thus already in a low-activity state, has little effect on the activity against benzylpenicillin. On complete hydrolysis of the quinacillin, however, the enzyme does not now regain even its normal antibody-reduced activity. It is 'locked-in' to the low-activity form by the antibody (see figure 1).

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The extent of conformational change was investigated by fast quantitative precipitin experiments. These show that *more* antibody (1.5 times) is bound by the enzyme in its deactivated state than in its native state. This indicates not only an appreciable change in conformation but also the exposure of antigenic sites not normally 'seen' in the native enzyme.

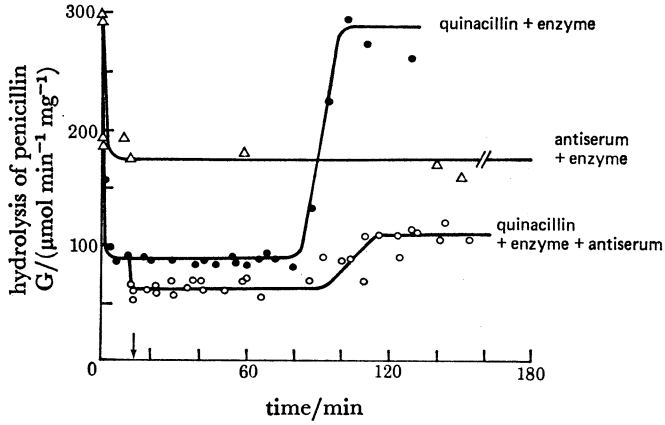

FIGURE 1. The effect of antiserum on the recovery of benzylpenicillinase activity from staphylococcal β-lactamase incubated with quinacillin. Quinacillin (5 mM) incubated with β-lactamase (150 μg ml^{-1}) and enzyme activity determined by dilution into benzylpenicillin solutions (●); rabbit anti-β-lactamase added after 12 min preincubation with quinacillin (○); β-lactamase with antiserum alone (△).

The β-lactamase from *S. aureus*, it has been suggested, has a three-domain structure in which the domains are rather loosely (ΔG = 10 kJ mol^{-1}) bonded together (Carrey & Pain 1978). Under physiological conditions, therefore, the interdomain faces of 1 molecule in 30 will be exposed at any one time and thus allow antibodies to be formed against these antigenic sites. If, on the basis of many precedents, we assume that the active site groups are contributed by more than one domain, then the activity of the enzyme will depend in a sensitive way on the orientation of the domains. We propose that reversible deactivation occurs when quinacillin modifies this orientation and in doing so exposes antigenic sites (Pain & Virden 1979).

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7. Tertiary and secondary structure analysis of penicillin-binding proteins

By M. L. DeLucia, J. A. Kelly, M. M. Mangion, P. C. Moews and J. R. Knox
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[Plate 1]

The design of enzyme-resistant β-lactams and β-lactamase inhibitors will be aided by mapping the structure of enzyme receptors (D-alanyl-carboxypeptidases–transpeptidases) and β-lactamases, especially near the sites of substrate binding. That the two types of enzyme are probably structurally related has just been shown at this meeting by Strominger, who demonstrated a 30–40% sequence homology in *N*-terminal regions of β-lactamases and D-alanyl-carboxypeptidases. Though both contain a serine site reactive to β-lactams, the reaction product is different in each case, so important structural differences in penicillin-binding sites must exist in the two types of enzyme. To define these differences and to visualize the interaction of these enzymes with β-lactams, we are determining their three-dimensional structures by X-ray diffraction.

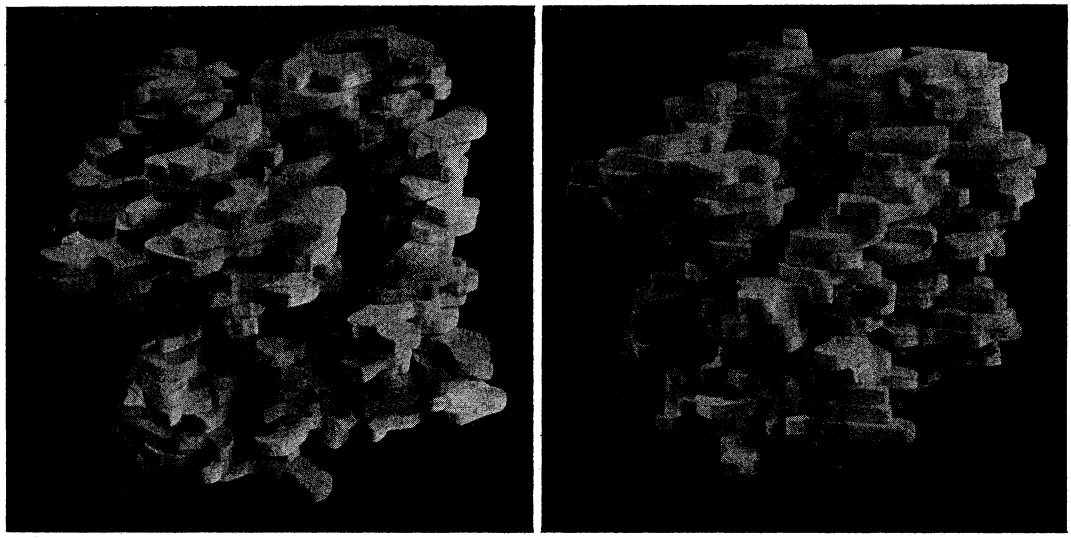

FIGURE 1. Views of the R_{TEM} β-lactamase model at 4 Å resolution. The molecular diameter is about 40 Å.

Our X-ray analysis of the *Escherichia coli* R_{TEM} β-lactamase at 4 Å† resolution has recently been reported (Knox *et al.* 1979), and we show in figure 1 several views of the current model. While establishing the general shape and size of the molecule, these results unfortunately reveal, at this time, little of biochemical interest. However, several presumed inhibitors such as clavulanic acid, 6-β-bromopenicillanic acid and various sulphones, are being soaked into pregrown crystals to allow location and examination of the binding site. As the X-ray analysis continues towards 2.5 Å resolution, the primary sequence (Ambler, this volume) will be fitted

† 1 Å = 10^{-10} m = 10^{-1} nm.

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to the electron density map and the reactive serine 44 (Waley, this volume) will be positioned within the model.

To compare the R_{TEM} β-lactamase structure with those from other species, we have used two statistical methods to predict secondary folding (Moews & Knox 1979). Both methods strongly predict that the R_{TEM} structure should be very similar to the structures of three enzymes from the Gram-positive *Bacillus cereus*, *Bacillus licheniformis* and *Staphylococcus aureus*. All four β-lactamases are expected to be of the α/β type (class IV of Levitt & Chothia 1976), with helices at *N*- and *C*-termini. There are about seven short regions each of α (20–40 %) and β-strand (10–20 %) structure separated by 20–25 reverse turns in homologous positions in all four enzymes (figure 2). The catalytically implicated serine 44 is in a generally helical area for the *B. licheniformis* and R_{TEM} enzymes, while for the *S. aureus* and *B. cereus* enzymes the structure prediction around the serine is ambiguous.

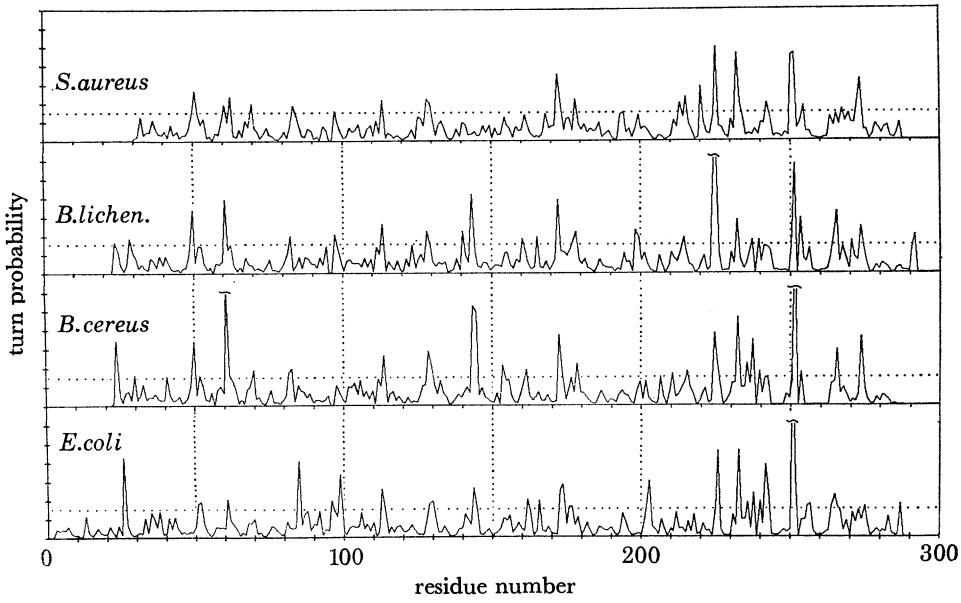

FIGURE 2. Probability of reverse turn plotted against residue number for four β-lactamases. For primary sequences, see Ambler, this volume.

X-ray analysis of a penicillin receptor is well underway. The penicillin-sensitive DD-carboxypeptidase–transpeptidase from *Streptomyces* R61 has been crystallized from polyethylene glycol solution at pH 7.6 (Knox *et al.* 1979). X-ray examination of the orthorhombic crystals shows that the space group is $P2_12_12_1$, with unit cell dimensions a = 51.1 Å, b = 67.4 Å, and c = 102.9 Å (figure 3, plate 1). With four molecules of molecular mass 38 000, the ratio of volume to molecular mass for the cell is 2.33 Å^3. Native diffraction data have been collected to 5 Å resolution, and it is estimated that the data are measurable to at least 2.5 Å resolution. The useful crystal lifetime is about 100 h. Data have now been collected from a very isomorphous uranyl derivative, and an electron density map of the molecule has been calculated to 5 Å resolution (figure 4, plate 1). Penicillin and cephalosporin complexes of the receptor are being subjected to X-ray analysis. A very recent and preliminary result is seen in figure 4*b*, which shows the difference density attributable to the effects of *o*-iodophenylpenicillin on the receptor

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structure. This study must be done at higher resolution before details of β-lactam binding can be given. Similar crystallographic binding studies with D-alanyl-D-alanine substrates are planned.

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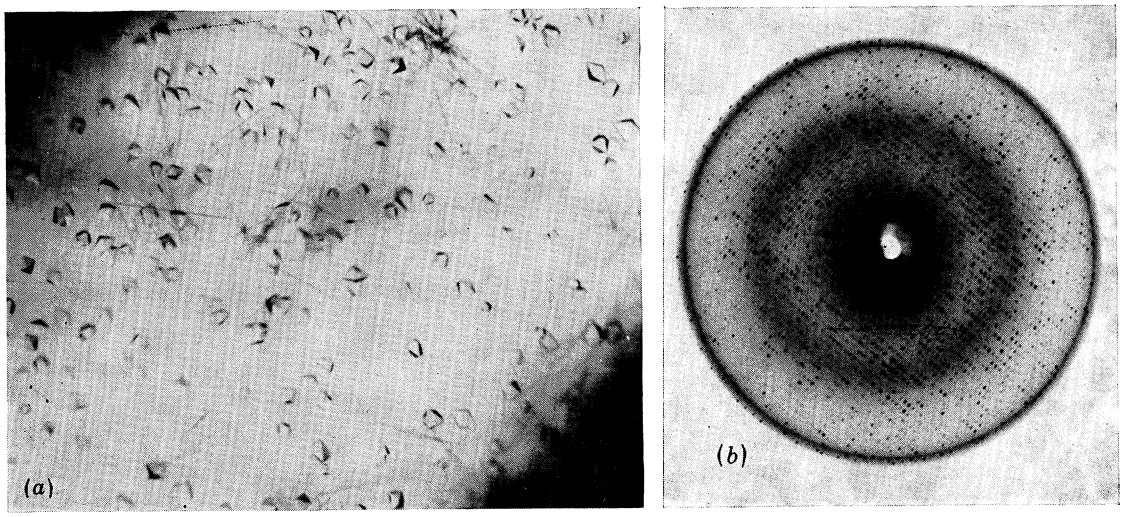

FIGURE 3. (*a*) Crystals of DD-carboxypeptidase–transpeptidase from *Streptomyces* R61. Growth occurs in 15–20 % polyethylene glycol (molecular mass 6000) at room temperature. (*b*) X-ray diffraction photograph of 0*kl* zone to 2.5 Å resolution.

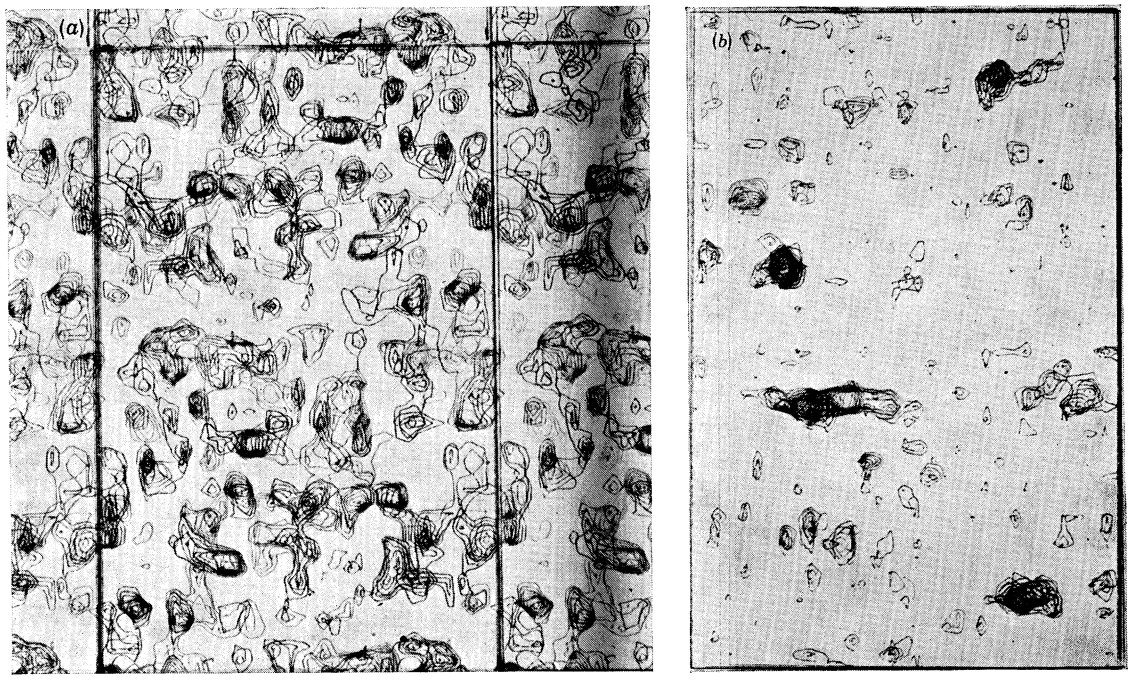

FIGURE 4. (*a*) Selected *yz* sections of electron density of DD-carboxypeptidase–transpeptidase at 5 Å resolution. (*b*) Electron density *difference* between native crystal and one soaked in 40 mM *o*-iodophenylpenicillin. These sections are near those in figure 4*a*. Two peaks on left are significantly above background (two peaks on right are their symmetry mates).

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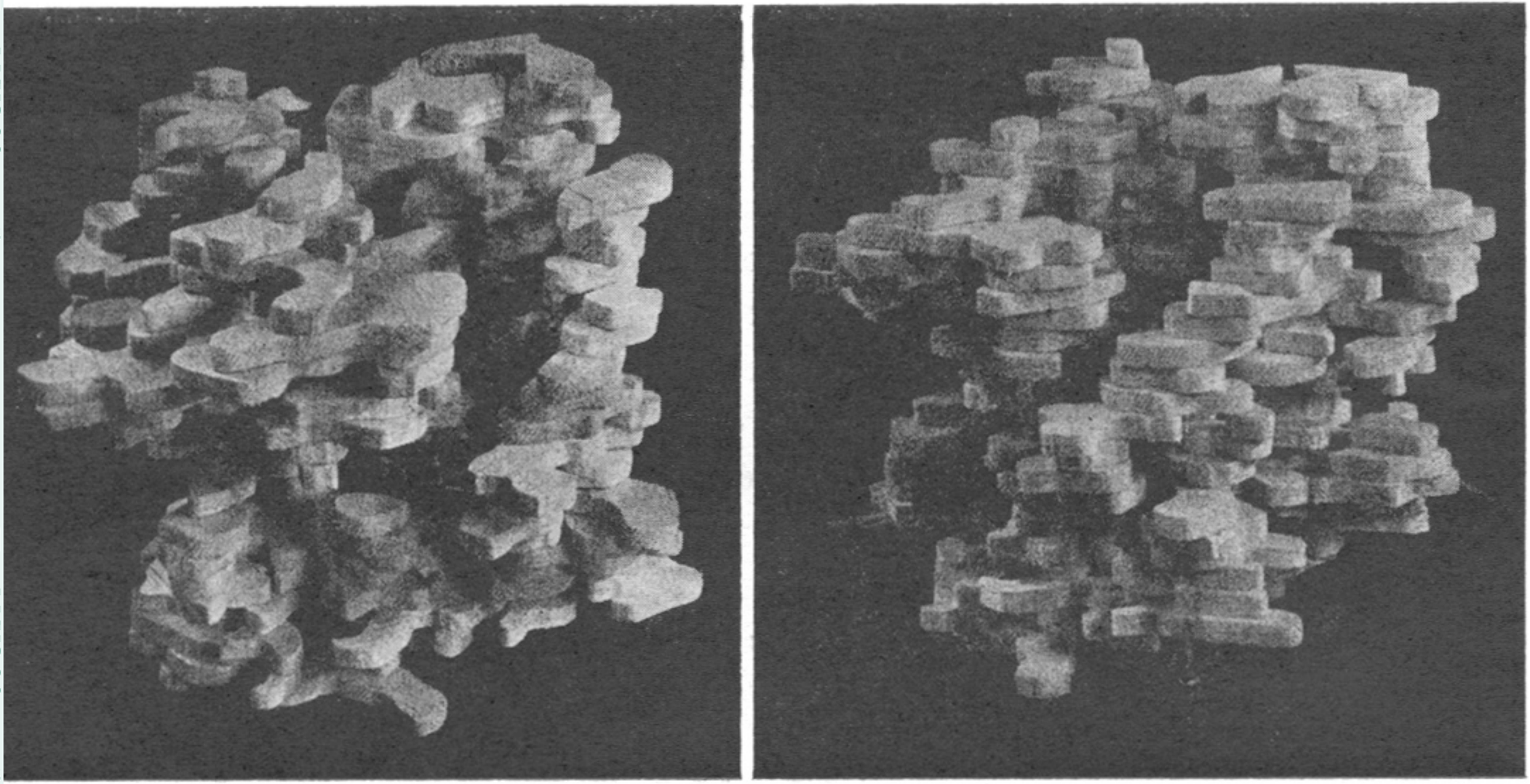

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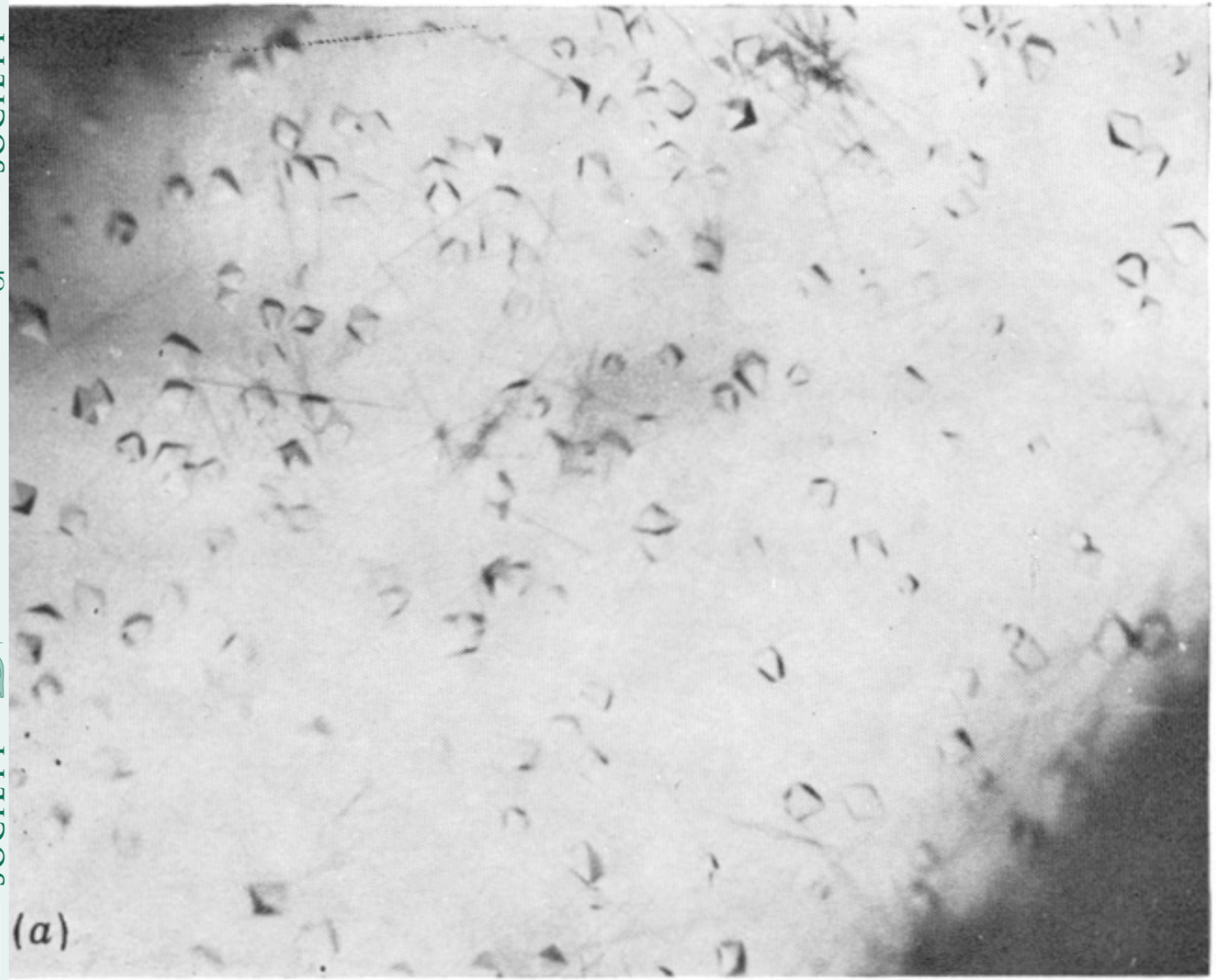

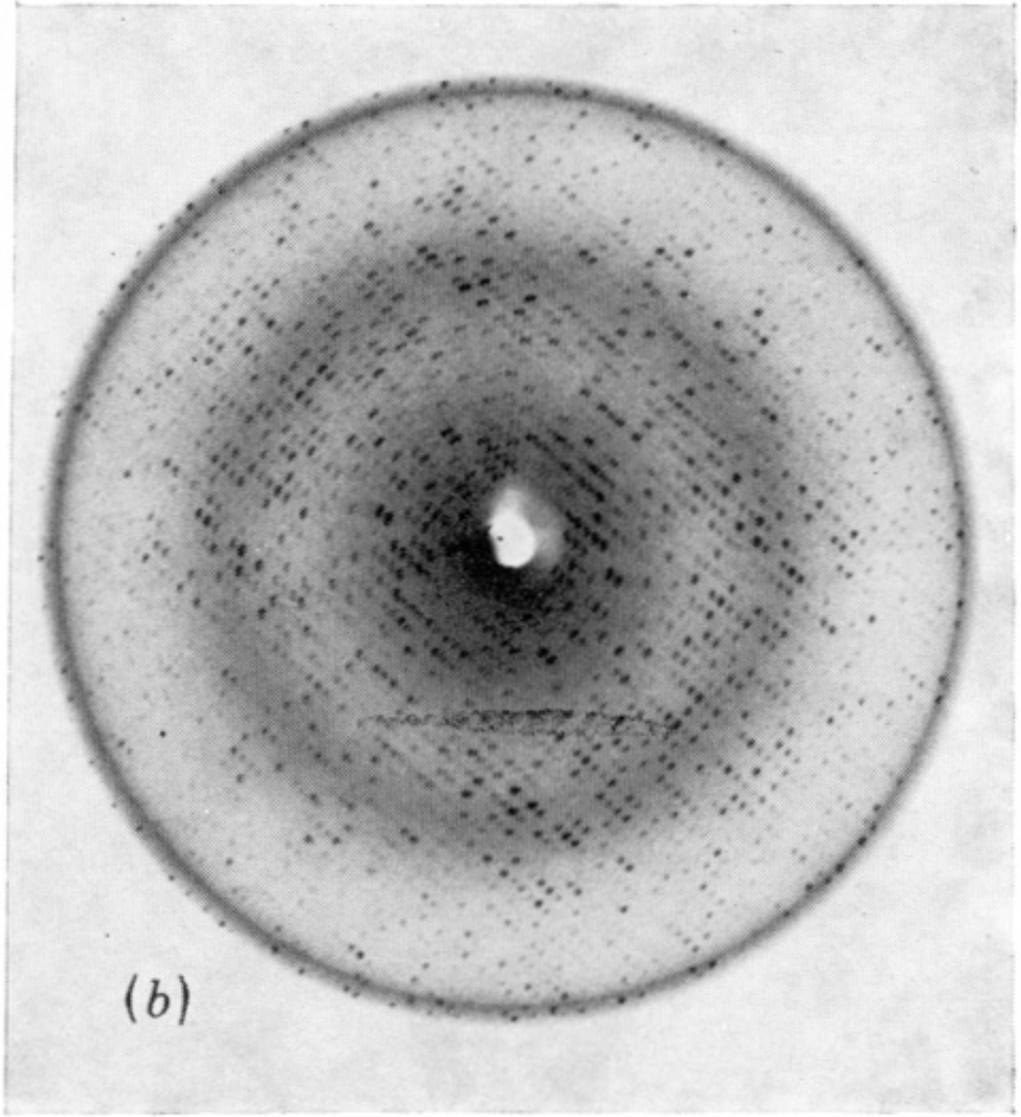

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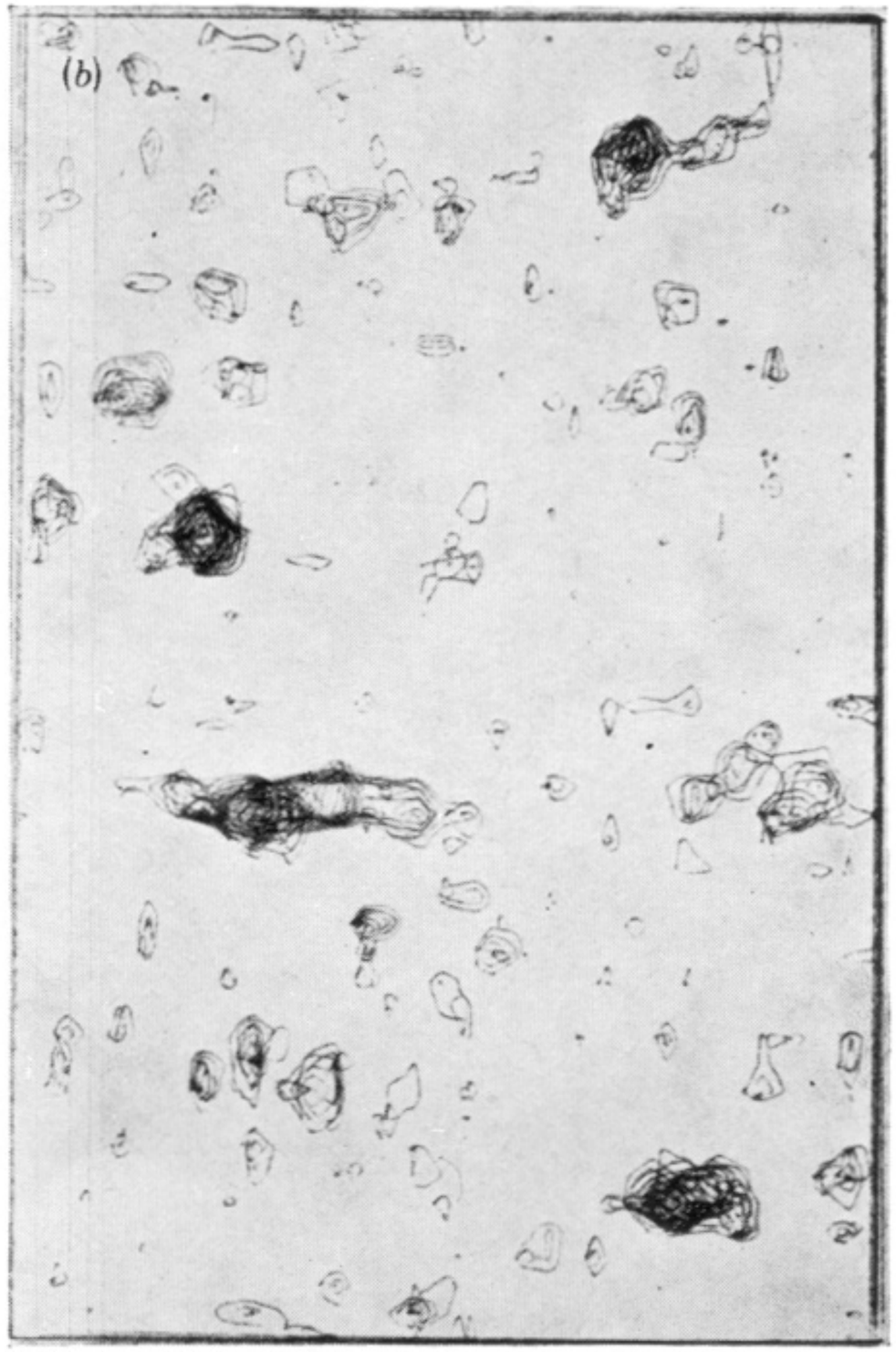

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